

The C-terminus of pokeweed antiviral protein has distinct roles in transport to the cytosol, ribosome depurination and cytotoxicity

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Summary

Pokeweed antiviral protein (PAP) produced by pokeweed plants is a single-chain (type I) ribosome-inactivating protein (RIP) that depurinates ribosomes at the α -sarcin/ricin loop of the large rRNA, resulting in inhibition of translation. Unlike the type II RIPs, which have an active and a binding moiety, PAP has only the active moiety. The mechanism by which toxins without a binding moiety gain access to cytosolic ribosomes is not known. We set up yeast as a simple and genetically tractable system to investigate how PAP accesses ribosomes and showed that the mature form of PAP is targeted to the cytosol from the endomembrane system in yeast. In the present study, we performed a systematic deletion analysis to identify the signal required for transport of PAP to the cytosol. We demonstrate here that processing of the C-terminal extension and sequences at the C-terminus of the mature protein are critical for its accumulation in the cytosol. Using a series of PAP mutants, we identified the C-terminal signal and demonstrated that it is distinct from the sequences required for ribosome depurination and cytotoxicity. The C-terminal motif showed sequence similarity to type II RIPs that retrotranslocate from the endoplasmic reticulum to the cytosol. These results demonstrate that a conserved sequence at the C-terminus of a type I RIP mediates its transport to the cytosol and suggest that type I and II RIPs may use a common signal to enter the cytosol.

Keywords: pokeweed antiviral protein, ribosome-inactivating protein, retrotranslocation, ribosome depurination, translation inhibition.

Introduction

Ribosome-inactivating proteins (RIPs) such as ricin, Shiga toxin and pokeweed antiviral protein (PAP) catalytically remove an adenine (A4324) from the universally conserved sarcin/ricin loop (SRL) of the large rRNA, inhibiting the elongation step of protein synthesis (Barbieri *et al.*, 1993; Endo *et al.*, 1987). Pokeweed antiviral protein is a single chain or type I RIP isolated from the leaves of the pokeweed plant (*Phytolacca americana*). The plant toxins ricin and abrin and the bacterial toxins Shiga and Shiga-like toxins are type II RIPs with an active (A) chain homologous to the type I RIPs and one or more binding (B) chains, which bind to cell surface receptors (Hartley and Lord, 2004; Stirpe, 2004). The AB-toxins have provided us with essential information about mechanisms of entry into cells and intracellular trafficking pathways. The depurination of the 28S rRNA by RIPs leads to activation of apoptotic pathways

(Nielsen and Boston, 2001). Because of their ability to induce apoptosis, the catalytic subunits of RIPs have been used in cancer therapy as the active moiety of immunotoxins that selectively target cancer cells (Bolognesi and Polito, 2004; FitzGerald *et al.*, 2004; Frankel *et al.*, 2000). Their potent toxicity and wide availability have been exploited as weapons and as agents of bioterrorism (Knight, 1979). Bacteria producing Shiga-like toxins are the most common cause of hemolytic uremic syndrome (HUS), a disease for which there is no vaccine or effective treatment (Paton and Paton, 1998). Unlike the type II RIPs, type I RIPs have potent antiviral activity and confer broad-spectrum resistance to plant and animal viruses (Parikh and Tumer, 2004; Wang and Tumer, 2000).

Ribosome-inactivating proteins are synthesized as precursors and are compartmentalized to ensure that the

ribosomes of the plants that produce them never encounter them, leaving overall protein synthesis unaffected. Pokeweed antiviral protein is localized in the cell wall of *P. americana* leaves (Lodge *et al.*, 1993; Ready *et al.*, 1986). The A (RTA) and B (RTB) subunits of ricin are synthesized together as part of a single precursor. The ricin holotoxin, which contains the RTA and the RTB covalently joined together by a disulfide bond, accumulates in the protein storage vacuoles in *Ricinus communis* seeds (Hartley and Lord, 2004). The RTA subunit becomes active only after cleavage and release from RTB (Richardson *et al.*, 1989). Pokeweed antiviral protein and other type I RIPs are synthesized as precursors with an N-terminal signal sequence and a C-terminal extension with an unidentified role in targeting (Nielsen and Boston, 2001; Parikh and Tumer, 2004).

The bacterial and plant protein toxins that disrupt protein synthesis have cytosolic targets, so at least a portion of the toxin must cross a cellular membrane. The type II RIPs, such as Shiga, Shiga-like toxins and ricin, do not disrupt cellular membranes directly. After binding their receptors on the surface of animal cells, they travel to the endoplasmic reticulum (ER) and are thought to enter the cytosol using the ER-associated degradation pathway (ERAD) (Lord *et al.*, 2003; Rapak *et al.*, 1997; Sandvig *et al.*, 1992; Simpson *et al.*, 1999). Since ERAD is normally used to dislocate misfolded proteins from the ER to the cytosol for destruction by the ubiquitin proteasome pathway, toxins must evade complete degradation by this pathway to carry out their enzymatic action in the cytosol (Lord *et al.*, 2003; Sandvig and van Deurs, 2005).

There is evidence that proteins associated with the ERAD machinery in yeast and animals are also conserved in plants (Kirst *et al.*, 2005; Martinez and Chrispeels, 2003). A misfolded barley protein, MLO-1 has been shown to be a substrate for the ERAD pathway in plants (Muller *et al.*, 2005). Studies with ricin A chain in transformed tobacco protoplasts provided evidence for the existence of protein retrograde transport and degradation pathways in plant cells that are similar to those in mammalian cells (Di Cola *et al.*, 2001). Very little is known about the mechanism by which the catalytic domains of type I RIPs reach cytosolic ribosomes. Transgenic plants expressing PAP showed a reduction in growth and lesions on their leaves (Lodge *et al.*, 1993). Ribosomes were depurinated in these plants, indicating that PAP was able to enter the cytosol (Tumer *et al.*, 1997).

Because expression of wild-type PAP is very toxic to transgenic plants (Lodge *et al.*, 1993), we have established yeast as a simple and genetically tractable system for investigating the biological activity of PAP and have shown that the yeast system faithfully reproduces the entry of PAP to the cytosol, depurination of cytosolic ribosomes, translational arrest and cell death (Hudak *et al.*, 2004; Parikh *et al.*, 2002, 2005). The precursor and the mature form of PAP are

localized in the endomembrane system in yeast (Parikh *et al.*, 2005). Pulse chase analysis showed that the precursor form is rapidly targeted to the membrane fraction and the mature form, which is processed at its N- and C-termini, accumulates in the cytosol (Parikh *et al.*, 2005). These results indicate that PAP traverses an internal membrane, thought to be the ER membrane, to enter the cytosol (Parikh *et al.*, 2005). The signals that RIPs use to reach cytosolic ribosomes have not been characterized. Studies with Shiga-like toxin I in yeast suggested that a misfolded or an altered peptide domain at the C-terminus of the A1 subunit, rather than recognition of a specific sequence element, was involved in its export from the ER (LaPointe *et al.*, 2005). Here, using a series of PAP mutants, we investigated the signal required for entry of PAP to the cytosol and identified a C-terminal motif, which is distinct from the sequences required for catalytic depurination of ribosomes and cytotoxicity. This motif shows sequence similarity to other type I and to type II RIPs, suggesting that type I and type II RIPs may use a common recognition signal to enter the cytosol.

Results

*Processing of PAP to its mature form is affected in V73E and L252**

Pokeweed antiviral protein is synthesized in pokeweed plants as a 313-amino-acid precursor with a 22-amino-acid N-terminal signal sequence and a 29-amino-acid C-terminal extension as depicted in Figure 1. Both sequences are processed to generate the mature form, which consists of 262 amino acids (1–262). Structure–function analysis of PAP identified a highly conserved stretch of amino acids outside the active site which are critical for cytotoxicity (Hudak *et al.*, 2004). Mutation of Val73 to Glu (V73E) in the central domain eliminated the cytotoxicity of PAP (Hudak *et al.*, 2004).

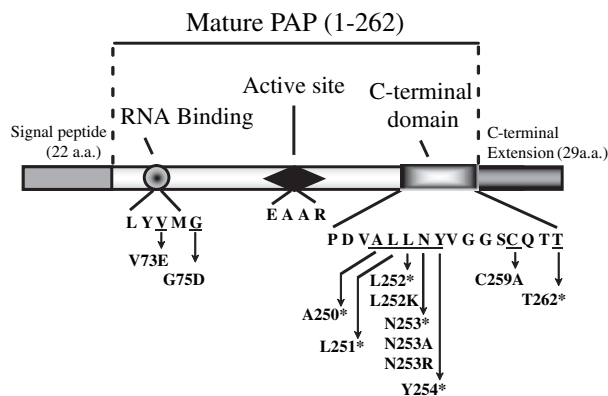


Figure 1. Diagram of wild-type PAP showing the mutated residues. The precursor form of PAP (313 amino acids) contains a 22-amino-acid signal sequence at its N-terminus and a 29-amino-acid C-terminal extension. The mature protein is 262 amino acids in length (1–262). The amino acids that are mutated in this study are indicated (*, stop codon).

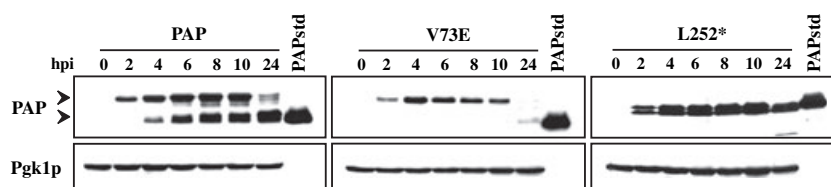


Figure 2. Immunoblot analysis of PAP and PAP mutants.

Yeast cells were induced for the hours indicated (hpi). Total protein (10 µg) from each time point was separated on a 15% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with polyclonal anti-PAP serum. Purified PAP (10 ng) from pokeweed leaves was used as the standard. The blots were subsequently stripped and reprobed with anti-Pgk1p antibody as a loading control. The arrows indicate the precursor and the mature forms of PAP.

Similarly, cytotoxicity was abolished when a stop codon was introduced at Leu252 (L252*), deleting the C-terminal extension and 11 amino acids from the C-terminus of the mature protein (Hudak *et al.*, 2004). A possible explanation for the loss of cytotoxicity in V73E and L252* is that these mutations may have affected maturation of PAP and its ability to retrotranslocate to the cytosol. To determine whether processing of PAP is affected, we examined accumulation of the precursor and the mature forms of PAP in yeast expressing the wild-type PAP, V73E and L252*. As shown in Figure 2, the protein level corresponding to the mature form of PAP increased and the precursor form of PAP decreased with time in cells expressing the wild-type PAP. By 24 h post-induction most of the protein in the cell corresponded to the mature form. Only the precursor form of PAP was observed in cells expressing V73E. This form did not increase in abundance after 4 h of induction. Instead, its abundance decreased at 24-h post-induction and a very low level of the mature form was observed, suggesting that the mutation at Val73 affected processing and stability of the precursor form. The L252* consisted of a doublet, which migrated slightly faster than the PAP standard (Figure 2). The precursor form was not observed in this mutant, since a total of 40 amino acids were deleted from the C-terminus. The blots were stripped and reprobed with antibody against phosphoglycerate kinase (Pgk1p) as a loading control.

C-terminal processing is critical for accumulation of the mature form in the cytosol

Since V73E and L252* were not processed as the wild-type PAP in yeast, their subcellular localization might be altered. We examined the subcellular localization of V73E and L252* by fractionating yeast extracts into cytoplasmic and membrane fractions. Both fractions were analyzed for the presence of V73E and L252* by immunoblot analysis at different times after induction. The antibodies for the cytosolic protein, Pgk1p and the integral ER membrane protein dolichol phosphate mannosyl synthase (Dpm1p) were used to confirm the lack of cross-contamination between the cytoplasmic and the membrane fractions. As shown in Figure 3(a), the precursor and the mature form of wild-type PAP are associated with the membrane and the cytosolic fractions as previously

reported (Parikh *et al.*, 2005). The precursor form observed in the cytosol by steady-state analysis is not transported there, but may reflect cytoplasmic overexpression, since PAP appears to be transported to the ER in a post-translational manner (Parikh *et al.*, 2005). The abundance of the precursor form decreased and the abundance of the mature form increased in the membrane and in the cytosol by 24-h post-induction. In contrast to wild-type PAP, only the precursor form was detected in cells expressing V73E, and this form co-fractionated exclusively with the ER marker Dpm1p. These results suggested that the Val73 mutation affected processing of the C-terminal extension and resulted in accumulation of the protein in the membrane fraction. A decrease in accumulation of V73E in the membrane fraction was observed at 24 h after induction. However, the mature form was not detected. These results indicated that the processing of V73E was inhibited and suggested that its stability was reduced. Similarly, deletion of the C-terminal extension and 11 amino acids from the C-terminus of the mature protein in L252* resulted in accumulation of the truncated protein primarily in the membrane fraction (Figure 3a).

We analyzed another mutation in the highly conserved region near Val73 to determine if it affected processing and subcellular localization of PAP. Previous results showed that G75D is not cytotoxic, does not depurinate ribosomes and does not inhibit translation (Hudak *et al.*, 2004). As shown in Figure 3(b), only the precursor form was observed in yeast expressing G75D and it co-fractionated exclusively with the membrane fraction (Figure 3c). The mature form of PAP was not observed in either the membrane or the cytosolic fraction in cells expressing G75D. These results provided evidence that Val73 and Gly75 are critical for processing of the C-terminal extension and suggested that inhibition of C-terminal processing of the PAP precursor prevented export of the mature form to the cytosol. To determine if the C-terminally processed form of PAP is able to enter the cytosol, we introduced a nonsense mutation, deleting the last amino acid of the mature protein, Thr262, and the C-terminal extension (T262*). As shown in Figure 3(b), only the mature form of PAP was produced in yeast expressing T262*. Subcellular fractionation showed that T262* was present in both the membrane fraction and the cytosol (Figure 3c), indicating that the mature form of PAP without

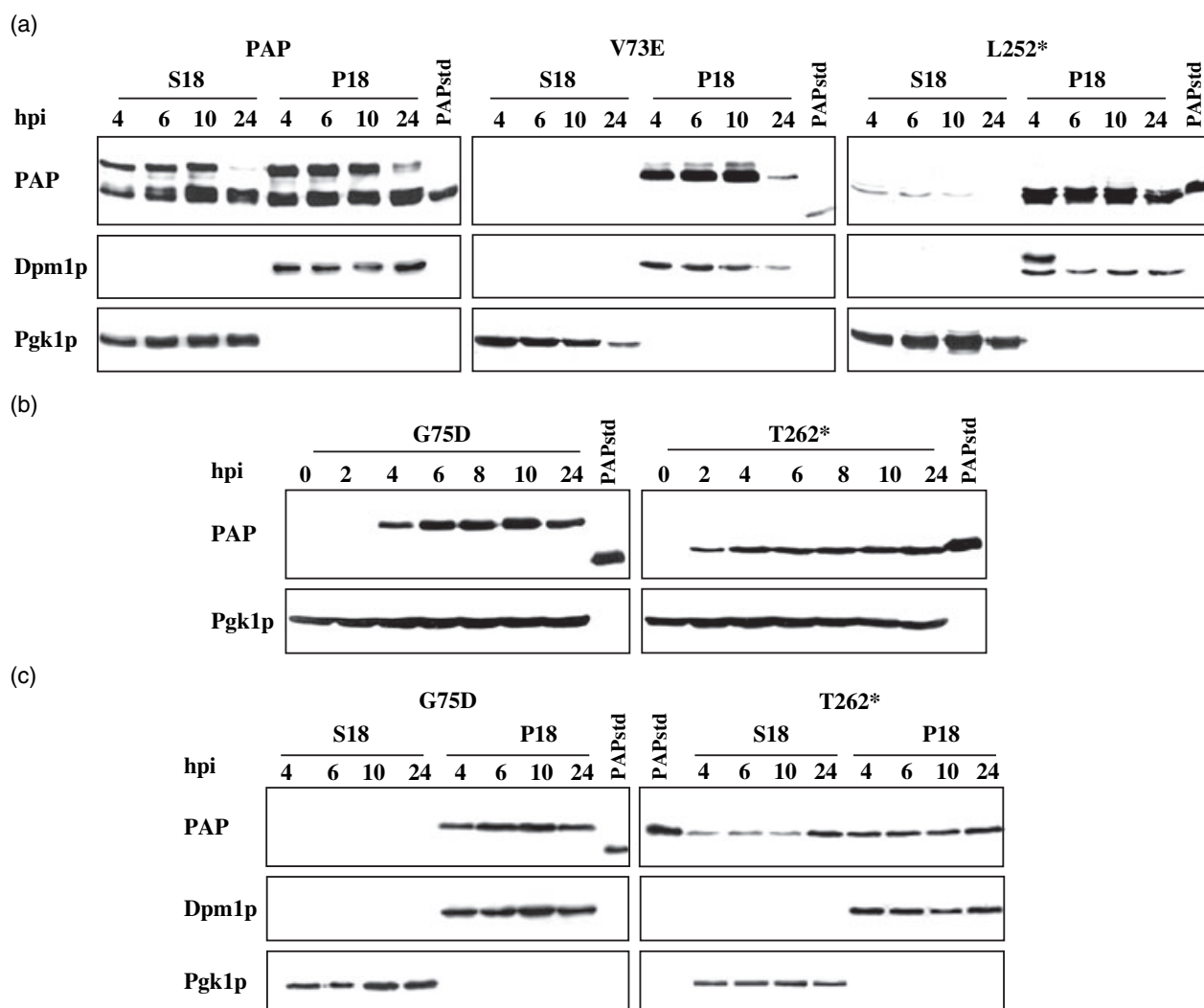


Figure 3. Steady-state accumulation of PAP and the PAP mutants in the membrane and cytosolic fractions.

(a) Yeast cells were induced for the hours indicated (hpi), lysed and fractionated into membrane (P18) and cytosolic (S18) fractions. Total protein (10 µg) from each time point was separated on a 15% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with polyclonal anti-PAP serum. Antibodies against the ER membrane marker, Dpm1p, and the cytosolic marker, Pgk1p, were used as controls to demonstrate the lack of cross-contamination.

(b) Yeast cells were induced for the hours indicated and total protein (10 µg) from each time point was separated on a 15% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with polyclonal anti-PAP serum. The blots were subsequently stripped and reprobed with anti-Pgk1p antibody as a loading control.

(c) Yeast cells were induced for the hours indicated, lysed and fractionated into membrane (P18) and cytosolic (S18) fractions. Total protein (10 µg) from each time point was separated on a 15% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with anti-PAP serum. Antibodies against the ER membrane marker, Dpm1p, and the cytosolic marker, Pgk1p, were used as controls to demonstrate the lack of cross-contamination.

its C-terminal extension was able to enter the cytosol. These results were in agreement with the previous studies, which indicated that T262* was cytotoxic, depurinated ribosomes and inhibited translation (Hudak *et al.*, 2004).

V73E and L252 are localized on the luminal side of microsomal membranes*

Previous results indicated that V73E and L252* were localized primarily in the membrane fraction. To determine whether they are localized in the ER, we isolated microsomes from yeast expressing the wild-type and the mutant

forms of PAP. Microsomes were treated with proteinase K in the presence and the absence of the detergent Triton X-100 to investigate the distribution of V73E and L252* with respect to the luminal and cytosolic faces of microsomal membranes. As shown in Figure 4, the wild-type PAP, V73E and L252* were localized in the microsomes. In the absence of the detergent, proteinase K was not able to degrade the wild-type PAP or the mutant proteins, indicating that they were on the luminal side of the membrane and not accessible to protease K. The upper band of the doublet observed in the total extract from cells expressing L252* was absent from the microsomes, indicating that it

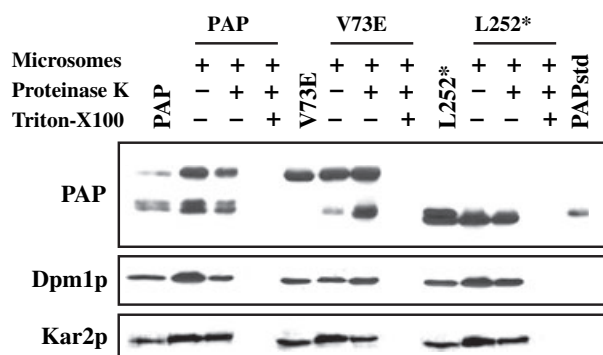


Figure 4. Proteinase protection assay.

Microsomes were isolated from yeast cells induced for 6 h and treated with buffer alone (microsomes), 100 µg ml⁻¹ proteinase K (Proteinase K) or 100 µg ml⁻¹ proteinase K and 2% Triton-X-100 (TritonX-100). After a 30-min incubation on ice, 5 mM PMSF was added to each sample and the samples were subjected to immunoblot analysis using anti-PAP serum. The blot was stripped and reprobed with anti-Dpm1p and anti-Kar2p. Lanes labeled PAP, V73E and L252* correspond to total protein from each lysate. Purified PAP (10 ng) was used as the standard.

was in the cytosol. In control experiments proteinase K digested the wild-type PAP in the cytosolic fraction in the absence of Triton X-100 (data not shown). Reprobing the blot with the anti-Kar2p or anti-Dpm1p showed that Kar2p, a yeast ER luminal protein, or Dpm1p, a yeast ER integral membrane protein, were not degraded by proteinase K in the absence of the detergent. However, in the presence of Triton X-100, wild-type PAP, V73E and L252* were degraded as Kar2p and Dpm1p. These results demonstrated transfer of the PAP mutants across the microsomal membrane. A low level of the mature form of V73E was detected in microsomes, and its abundance increased upon proteinase K treatment, suggesting that the C-terminal tail of V73E was susceptible to proteolysis.

C-terminal residues 250–262 are critical for accumulation of PAP in the cytosol

Since the mature form of PAP in T262* was transported to the cytosol, it contained the information necessary for export. To identify the sequence critical for export, we made sequential deletions from the C-terminus of mature PAP by introducing stop codons and examined the subcellular localization of the mutant proteins. As shown in Figure 5, deletion of Tyr254 and the sequences downstream (Y254*) retained the ability of PAP to accumulate in the cytosol. In contrast, deletion of Asn253 and the sequences downstream (N253*) reduced the accumulation of the protein in the cytosol and led to the appearance of a slightly larger form in the membrane fraction. Accumulation of PAP in the cytosol was reduced even more when Leu252 and the residues downstream were deleted (L252*), and was no longer detectable when a stop codon was introduced at Ala250

(A250*) (Figure 5). These results demonstrate that C-terminal residues 250–262 are critical for the accumulation of PAP in the cytosol. To determine whether the length of the C-terminal polypeptide chain between positions 250 and 262 or specific residues in this region are important, we introduced point mutations at Asn253 and Leu252, since deletion of these residues reduced the level of protein in the cytosol. As shown in Figure 5, changing Asn253 to alanine (N253A) or to arginine (N253R) did not affect accumulation of PAP in the cytosol, compared to the wild-type PAP (Figure 3). The point mutation at Leu252 (L252K), which changed this hydrophobic residue to a basic residue, caused a delay in the appearance of the mature form in the cytosol. L252K was detectable in the cytosol at 10 h after induction, in contrast to wild-type PAP, which was detected at 4 h post-induction (Figure 3). Unlike the wild-type PAP, the mutant protein did not accumulate in the membrane or in the cytosol at 24 h after induction, suggesting that its stability was reduced (Figure 5). When the L252K and N253A mutations were combined, the double mutant (L252K–N253A) accumulated in the cytosol up to 10 h, as observed with N253A. However, accumulation of the double mutant in the membrane and in the cytosol was reduced at 24 h, as observed with L252K (Figure 5). These results indicated that the N253A mutation did not completely overcome the effect of the L252K mutation when both mutations were combined.

Pokeweed antiviral protein contains two disulfide bonds, one between residues Cys34 and Cys259 and the other between Cys85 and Cys106 (Monzingo *et al.*, 1993). To determine whether formation of the disulfide bond between Cys34 and Cys259 is critical, we tested the ability of a point mutant at Cys259 (C259A) to accumulate in the cytosol. Processing of the C-terminal extension was affected in C259A, since two different processed forms were observed and the appearance of the mature form in the cytosol was delayed (Figure 5). These results indicated that the specific amino acids at positions 252 and 259 were critical.

Cytotoxicity of PAP mutants does not always correlate with their ability to enter the cytosol

We examined the viability of the C-terminal mutants at 10 h after induction to determine how the ability to enter the cytosol correlated with cytotoxicity. As shown in Figure 6(a), viability of yeast increased gradually upon sequential deletion of amino acids from the C-terminus of mature PAP. Deletion of Asn253 and the sequences downstream caused a significant increase in viability, which increased further upon deletion of Leu252. Just as the ability to accumulate in the cytosol was reduced when Asn253 was deleted, cytotoxicity also decreased.

As shown in Figure 6(b), yeast cells expressing the point mutants V73E and G75D were viable. Since the mutant

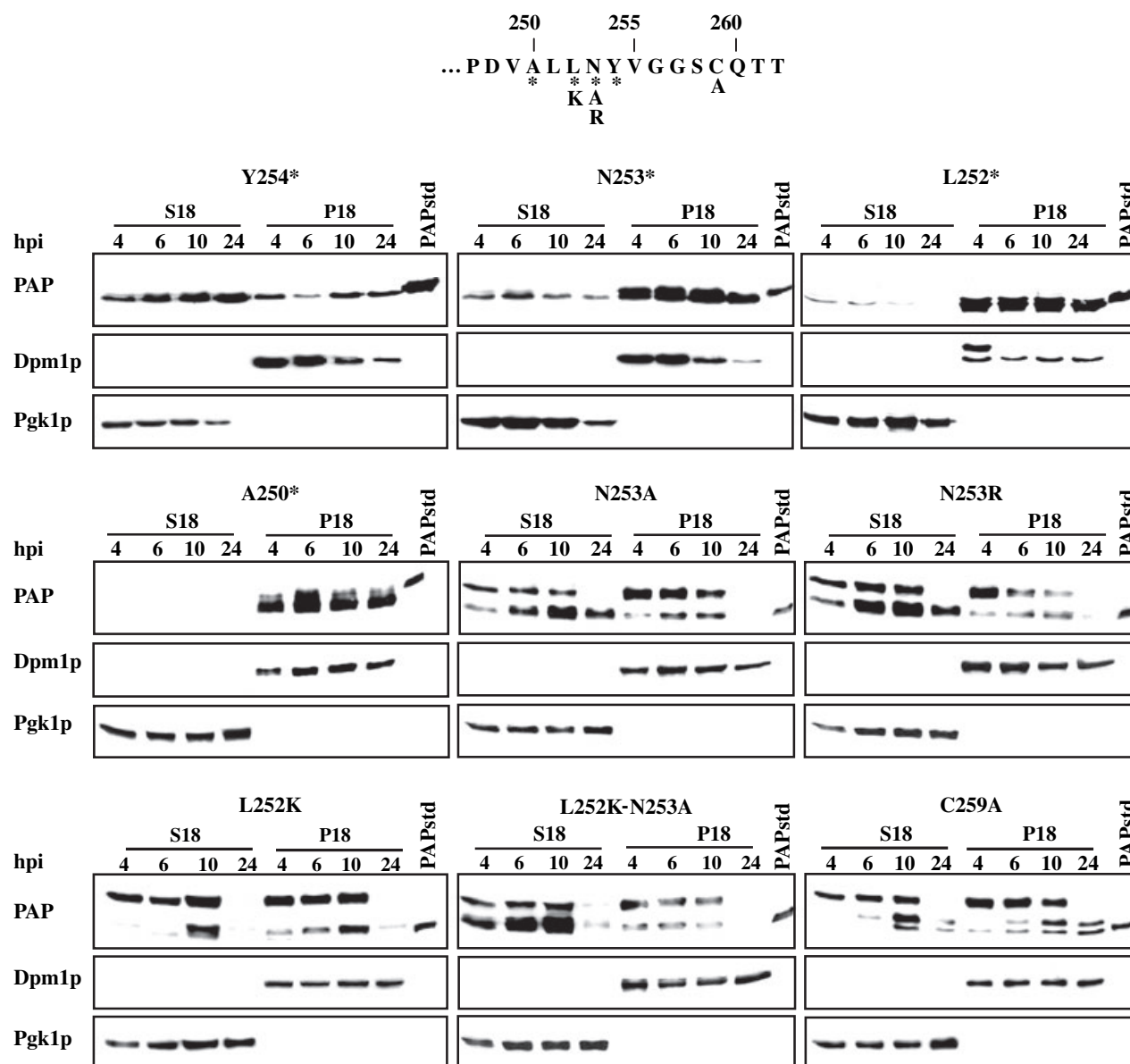


Figure 5. Steady-state accumulation of PAP mutants in the membrane and cytosolic fractions.

Yeast cells were induced for the hours indicated (hpi), lysed and fractionated into membrane (P18) and cytosolic (S18) fractions. Total protein (10 mg) from each time point was separated on a 15% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with polyclonal anti-PAP. Antibodies against the ER membrane marker, Dpm1p, and the cytosolic marker, Pgk1p, were used as controls to demonstrate the lack of cross-contamination. Purified PAP (10 ng) was used as the standard. The diagram at the top shows the position of the mutations in the C-terminal sequence of PAP. Data for L252* from Figure 3 were included for comparison with the C-terminal deletion mutants shown.

proteins were present only in the membrane fraction (Figure 3), their lack of cytotoxicity correlated well with their absence in the cytosol. The point mutations at Leu252 and Cys259, which delayed accumulation of the mature form, reduced viability like the wild-type PAP, possibly because the mutant proteins eventually entered the cytosol. Although the point mutations at Asn253 did not prevent accumulation in the cytosol, they increased viability. N253A, N253R and the double mutant, L252K-N253A, were not as toxic as the wild-type PAP, even though they accumulated in

the cytosol. To confirm that this effect was due to asparagine at position 253 and not the presence of the C-terminal peptide chain, we replaced Asn253 with Ala, Arg or Asp in the C-terminally truncated Y254* mutant, which was toxic. As shown in Figure 6(c), cytotoxicity of N253A-Y254* and N253R-Y254* was reduced. Substitution of aspartic acid for asparagine-253 in N253D-Y254* led to an even greater reduction in cytotoxicity (Figure 6c). These results demonstrated that asparagine at position 253 was critical for cytotoxicity, but not for accumulation in the cytosol, indica-

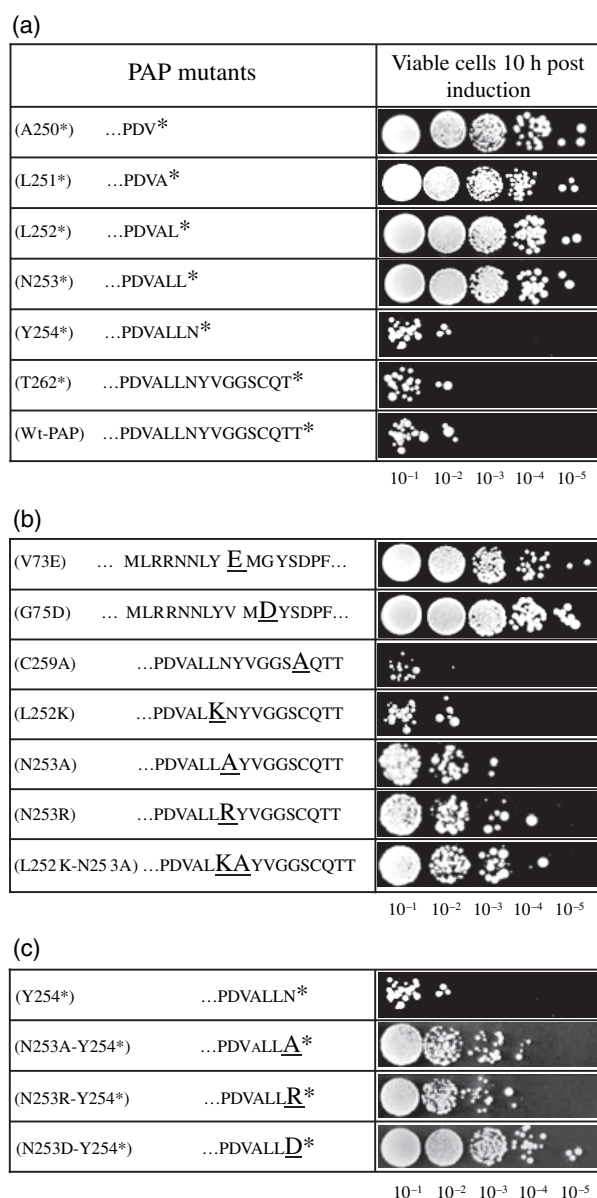


Figure 6. Viability of PAP mutants.

Cells were induced for 10 h in SD-Leu media containing galactose and serial dilutions were plated on non-inducing SD-Leu plates, containing glucose.

(a) Viability of the C-terminal deletion mutants.

(b) Viability of the point mutants.

(c) Viability of the Asn253 mutants.

The point mutations are underlined (*, stop codon).

ting that cytotoxicity of PAP does not always correlate with its ability to enter the cytosol.

Asparagine-253 is critical for catalytic depurination of ribosomes

The reduced cytotoxicity of the PAP mutants may be due to a reduction in the level of ribosome depurination relat-

ive to wild-type PAP. To determine how ribosome depurination is correlated with the ability to enter the cytosol, we isolated the PAP mutants from yeast and examined their ability to depurinate purified yeast ribosomes *in vitro* using a previously described dual primer extension assay (Parikh *et al.*, 2002). Immunoblot analysis of proteins used in the depurination assay is shown in Figure 7(a). Similar levels of the mutant proteins were isolated from yeast, except for V73E which was isolated at very low levels. The primer extension analysis of purified yeast ribosomes treated with the mutant proteins *in vitro* is shown in Figure 7(b) and is quantified in Figure 7(c). As previously observed, wild-type PAP isolated from yeast was able to depurinate yeast ribosomes *in vitro* (Parikh *et al.*, 2005) (Figure 7b). In contrast, L252* was not able to depurinate yeast ribosomes *in vitro*, while N253* caused an extremely low level of depurination. Since V73E was isolated at very low levels, two different concentrations were used in the depurination assay. The V73E depurinated yeast ribosomes at both concentrations, indicating that it was enzymatically active (Figure 7b,c).

The single or the double point mutations at Asn253 led to a large reduction in ribosome depurination *in vitro* (Figure 7b,c). Even though these proteins were extracted from yeast at higher levels than V73E, they could not depurinate yeast ribosomes as V73E *in vitro*. These results demonstrated that the mutations at Asn253, which led to a reduction in cytotoxicity, reduced the catalytic activity of the mutant proteins on yeast ribosomes *in vitro*. Although the mutant proteins accumulated in the cytosol, they could not depurinate ribosomes catalytically and consequently were less cytotoxic than the wild-type PAP.

Translation inhibition correlates with the ability to enter the cytosol and depurinate ribosomes catalytically

We have previously shown that wild-type PAP inhibits total translation by 70–75% at 4 h post-induction and translation remains inhibited after 4 h (Parikh *et al.*, 2002). To determine whether translation is inhibited in yeast expressing the non-toxic mutants V73E, L252* and N253* and the toxic mutant Y254*, we examined the incorporation of [³⁵S] methionine into total protein at 4 and 10 h post-induction and compared it with the level of inhibition observed in cells expressing the wild-type PAP. Translation rates were determined by measuring the slope of the curves shown in Figure 8 and compared to the active site mutant, E176V. Total translation was inhibited by 74% in cells expressing the wild-type PAP at 4 h post-induction and remained inhibited at 10 h. In contrast, total translation was not inhibited in cells expressing L252* at 4 h or at 10 h post-induction. Total translation was inhibited by only 24% in cells expressing N253* at 4 h post-induction and by 40% at 10 h post-induction. These results demonstrated that the

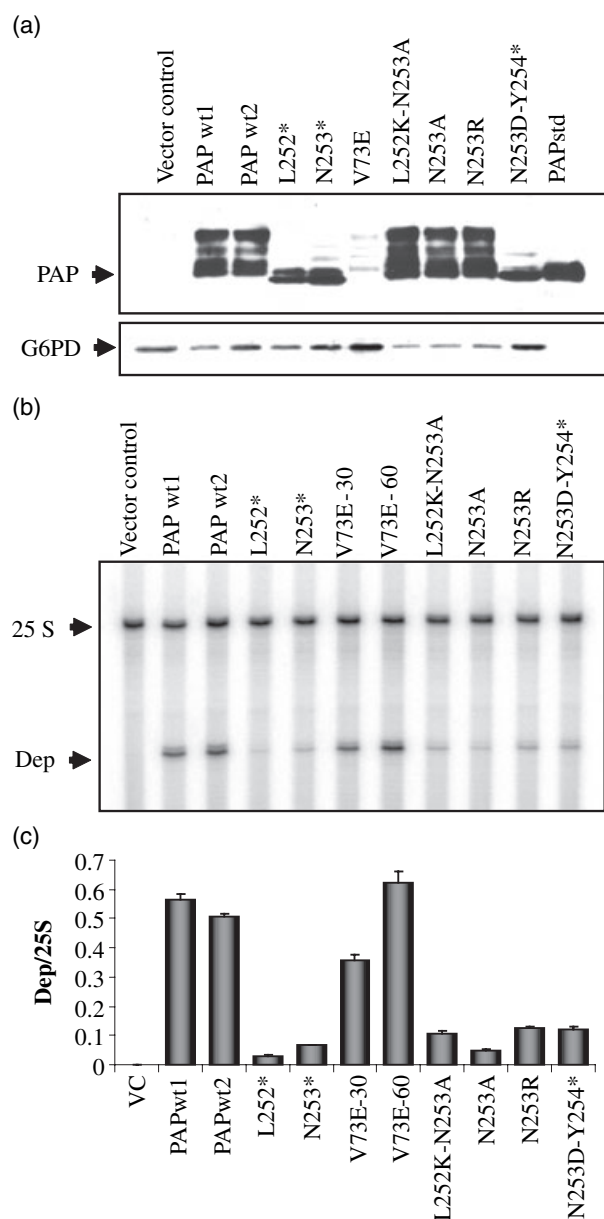


Figure 7. Ribosome depurination by PAP mutants *in vitro*.

(a) Immunoblot analysis of wild-type PAP and PAP mutants extracted from yeast at 6 h after induction. The wild-type PAP was extracted from two different colonies (wt1 and wt2). Total protein (10 µg) from L252*, N253* and N253D-Y254*, total protein (30 µg) from V73E and total protein (2.5 µg) from L252K-N253A, N253A and N253R was analyzed in each lane. Purified PAP (10 ng) was used as the standard. The blot was stripped and reprobed with anti-glucose-6-phosphate dehydrogenase (G6PD) as a loading control.

(b) The same amount of protein from wild-type PAP and PAP mutants used in the immunoblot analysis (a) was incubated with purified yeast ribosomes *in vitro*. Ribosome depurination was analyzed by primer extension using two different end-labeled primers, the depurination primer (Dep) used to measure the level of depurination and the 25S rRNA primer (25S) used to measure the amount of 25S rRNA.

(c) The extent of depurination was quantified by calculating the ratio of the depurination fragment (Dep) to the 25S rRNA fragment.

non toxic mutant L252*, which could not depurinate ribosomes catalytically, did not inhibit total translation, while N253*, which had a very low level of depurination activity, had reduced ability to inhibit total translation compared with the wild-type PAP. Total translation was inhibited by only 35% in cells expressing V73E, indicating that V73E did not inhibit total translation like the wild-type PAP at 4 h post-induction even though it was catalytically active. At 10 h post-induction translation was inhibited at a similar level (65%) in cells expressing V73E as in those expressing Y254*. These results demonstrated that translation inhibition correlated with the ability of the mutant proteins to enter the cytosol and catalytically depurinate the ribosomes.

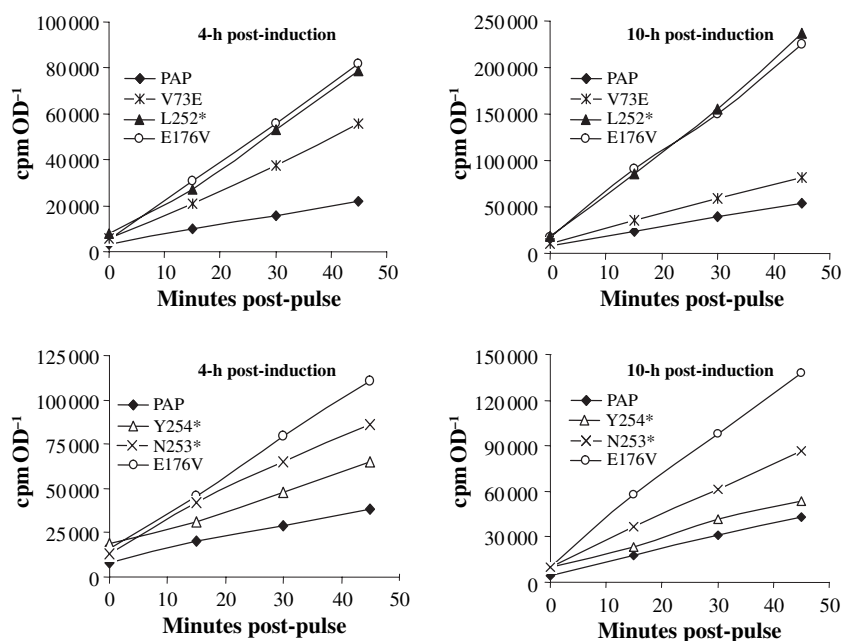
Discussion

C-terminal processing of the PAP precursor is necessary for export of PAP to the cytosol

A point mutation in valine-73 in the central domain outside the active site led to the loss of cytotoxicity of PAP in yeast. The mutant protein migrated slower than the mature form on the SDS-polyacrylamide gel, indicating that it was not fully processed. Unlike the wild-type PAP, which accumulated in both the membrane fraction and the cytosol, V73E accumulated only in the membrane fraction and not in the cytosol. Protease protection analysis of microsomes demonstrated that V73E was protected from proteolysis, indicating that it was on the luminal side of the microsomal membranes. A low level of the mature form of V73E was observed in microsomes and the abundance of the mature form increased upon protease treatment, suggesting that the C-terminal tail of V73E was exposed on the cytoplasmic side of the membrane. These findings provided evidence that the topology of V73E was slightly different from the wild-type PAP. The V73E extracted from yeast was able to depurinate ribosomes *in vitro*, indicating that the point mutation at Val73 did not cause a significant change in the structure of the active site. However, V73E did not inhibit translation *in vivo* at 4 h post-induction when maximal inhibition of translation occurred with the wild-type PAP, possibly because it lacked the ability to enter the cytosol. Consequently, V73E was not toxic to yeast cells. Translation was inhibited by 10 h post-induction, possibly because V73E depurinated ribosomes in a translation-dependent manner *in vivo* before it was sequestered into the ER (data not shown). These results demonstrated that mutations that affected transport of PAP to the cytosol also affected ribosome depurination and cytotoxicity, presumably due to lack of access of the mutant proteins to the cytosol.

The nonsense mutation at Leu252, which deleted 11 amino acids from the C-terminus of the mature protein, led to a dramatic reduction in cytotoxicity. The mutant protein

Figure 8. Analysis of total translation in yeast expressing the PAP mutants. Yeast harboring each plasmid was grown in SD-Leu-Met containing 2% galactose for 4 or 10 h to induce protein expression. At time zero, [35 S] methionine was added to cells growing on galactose and incorporation into total protein was determined at the indicated times. Each point was assayed in duplicate. The rates were determined for each construct in a minimum of two independent experiments.



accumulated only in the membrane fraction. The L252* extracted from yeast was not able to depurinate ribosomes *in vitro* as the wild-type PAP and did not cause any inhibition of total translation *in vivo*, indicating that it was not enzymatically active. These results demonstrated that deletion of 11 amino acids from the C-terminus of the mature protein eliminated its enzymatic activity and the ability to accumulate in the cytosol.

The deletion of the last amino acid and the C-terminal extension in T262* allowed the protein to enter the cytosol. However, this mutant did not accumulate in the cytosol as the mature form of wild-type PAP, suggesting that its stability was reduced. Similar results were obtained when the stop codon was introduced after T262, deleting only the C-terminal extension (data not shown). These results demonstrate that processing of the C-terminal extension is required for transport of the mature form of PAP to the cytosol and provide evidence that the C-terminus of the mature form contains the information necessary for export.

C-terminal residues of mature PAP are critical for its accumulation in the cytosol

Progressive deletion of amino acids from the C-terminus of mature PAP demonstrated that C-terminal residues 250–262 of the mature protein were critical for its accumulation in the cytosol. To determine whether the amino acid sequence of this region or the length of the polypeptide chain is important, we introduced point mutations at Leu252, Asn253 and Cys259. As observed with V73E, the point mutations at Leu252 and Asn253 reduced the accumulation of the protein in the membrane fraction. Substitution of hydrophobic res-

idues with a single charged residue within the transmembrane domain of integral membrane proteins has been shown to result in their rapid degradation in the ER (Bonifacino *et al.*, 1991). These observations suggest that substitution of Val73, Leu252 and Asn253 with the charged residues, may be responsible for the selective degradation of these proteins. Point mutations at Leu252 and Cys259 delayed export, but did not eliminate the cytotoxicity of the mutant proteins, possibly because they eventually entered the cytosol. Previous results indicated that L252K and C259A depurinated ribosomes and inhibited translation to a similar extent as the wild-type PAP (Hudak *et al.*, 2004), providing evidence that these mutations did not cause major alterations in structure. In contrast to mutations at Leu252 and Cys259, single mutations at Asn253 did not reduce the accumulation of PAP in the cytosol, indicating that the asparagine at position 253 was not critical for export. These results demonstrated that specific amino acids in the C-terminal domain between positions 250 and 262 are critical for the accumulation of PAP in the cytosol.

Asparagine-253 is critical for cytotoxicity, but not for accumulation in the cytosol

The nonsense mutation at Asn253 which decreased export led to a dramatic reduction in cytotoxicity. Similarly, point mutations at Asn253 either in the C-terminally truncated (1–253) or the full-length form of PAP (1–262), led to loss of cytotoxicity. These results demonstrated that Asn253, rather than the polypeptide chain between positions 253–262, was critical for cytotoxicity. To determine whether the loss of cytotoxicity was due to a defect in ribosome depurination,

we examined the ability of the mutant proteins to depurinate ribosomes *in vitro*. The N253* extracted from yeast caused an extremely low level of depurination *in vitro*. Analysis of total translation indicated that N253* did not inhibit translation to the same extent as the wild-type PAP. The point mutations at Asn253 reduced the ability of the protein to depurinate ribosomes catalytically. These results demonstrated that asparagine at position 253, rather than the C-terminal peptide chain, was critical for ribosome depurination. Modeling analysis indicated that Asn253, which is not directly involved in the catalytic depurination of the SRL, promotes specific interactions with the rRNA substrate (Rajamohan *et al.*, 2000). Thus, point mutations at this residue are predicted to disrupt stabilizing interactions with the phosphate backbone of the SRL, reducing the activity of the protein on ribosomes. We have previously shown that a point mutation at Asn70, which is predicted to interact with the phosphate backbone of the SRL, had a similar effect on the activity of protein on ribosomes (Parikh *et al.*, 2005). Substitution of the negatively charged aspartic acid in place of Asn253 resulted in the greatest reduction in cytotoxicity (Figure 6c), possibly by reducing the strength of binding to the negatively charged phosphate backbone of the rRNA. These results demonstrated that Asn253 at the C-terminus of mature PAP was critical for ribosome depurination and cytotoxicity, but not for transport to the cytosol, indicating that the ability to accumulate in the cytosol was not sufficient for cytotoxicity – the ability to depurinate ribosomes catalytically was also required.

We have previously shown that the ability of PAP to depurinate ribosomes decreased progressively with the deletion of C-terminal amino acids and cytotoxicity was lost before ribosome depurination was completely eliminated (Hudak *et al.*, 2004). The cytotoxicity of Asn253* was previously assayed by measuring its ability to grow on plates containing galactose and it was reported as cytotoxic, since it grows 1 day slower on galactose-containing plates compared to Leu252* (Hudak *et al.*, 2004). This difference was not as obvious when viability assays were conducted at 10 h post-induction (Figure 6), but became apparent at 24 h post-induction.

The hydrophobic C-terminal motif is conserved between the type I and the type II RIPs

The C-terminal residues critical for accumulation of PAP in the cytosol are part of a hydrophobic sequence at the C-terminus of the mature protein between residues 249–259, which is similar to the consensus sequence for a prokaryotic prolipoprotein binding motif (Hayashi and Wu, 1990). Analysis of the crystal structure of PAP indicated that these residues are not buried inside the toxin but are exposed on the surface of the protein (Monzingo *et al.*, 1993) (Figure 9a). Processing of the C-terminal extension of PAP may be

necessary to expose this sequence at the C-terminus of the mature protein, allowing its recognition by a chaperone or a receptor critical for export. Alternatively, the C-terminal sequence may bind to the membrane lipids, facilitating insertion of PAP into membranes. Pokeweed antiviral protein may unfold as a result of an interaction with the lipid membrane and the unfolded protein may be transported into cytosol.

Since the catalytically active A chains of type II RIPs, such as ricin and Shiga-like toxins, migrate from the ER lumen into the cytosol to exert their cytotoxic effect on eukaryotic cells, they may use a similar signal for retrotranslocation. As shown in Figure 9(b), the C-terminal hydrophobic peptide motif identified in PAP shows similarity to the C-terminal sequences of type II RIPs that enter the cytosol from the ER, as well as to other type I RIPs (Figure 9c). This sequence is normally buried inside the unprocessed form of the type II RIPs (Fraser *et al.*, 2004; Sixma *et al.*, 1993) and has been suggested to become exposed to solvent upon separation of the A and the B chains (Deeks *et al.*, 2002; Suhan and Hovde, 1998). A point mutation, which changed a conserved proline-250 to alanine at the C-terminus of ricin A chain reduced the cytotoxicity of the holotoxin (Simpson *et al.*, 1995). Since this mutation did not affect formation of the holotoxin, it was postulated to play a role in membrane interactions prior to retrotranslocation of the A chain into the cytosol (Simpson *et al.*, 1995).

A recent study published during the preparation of this manuscript examined retrotranslocation of Shiga-like toxin I using a similar approach in yeast, and suggested that the ER export mechanism involved recognition of a misfolded or altered peptide domain at the C-terminus of the A1 chain, rather than recognition of a specific sequence element (LaPointe *et al.*, 2005). The sequence identified in Shiga-like toxin I corresponds to the sequence deleted in N253* (Figure 9b), which is also critical for the cytotoxicity of PAP (Figure 6a). We show here that Asn253 is critical for the cytotoxicity of PAP, but not for its accumulation in the cytosol. These results indicate that cytotoxicity of RIPs does not always correlate with their ability to enter the cytosol.

Several components of the protein retrotranslocation and degradation pathways are conserved between yeast and plant cells (Kirst *et al.*, 2005; Martinez and Chrispeels, 2003; Muller *et al.*, 2005). Since the mutants characterized here, such as V73E, L252*, N253*, N253D–Y254*, N253A–Y254* and N253R–Y254*, which are not cytotoxic to yeast, are also not toxic when expressed in transgenic plants (UB and NET, unpublished data), we anticipate that the signals involved in protein export to the cytosol may be conserved between yeast and plants. We show here that processing of the C-terminal extension of the PAP precursor is critical for intracellular targeting of the mature form across an internal membrane in yeast. Although our results are consistent with

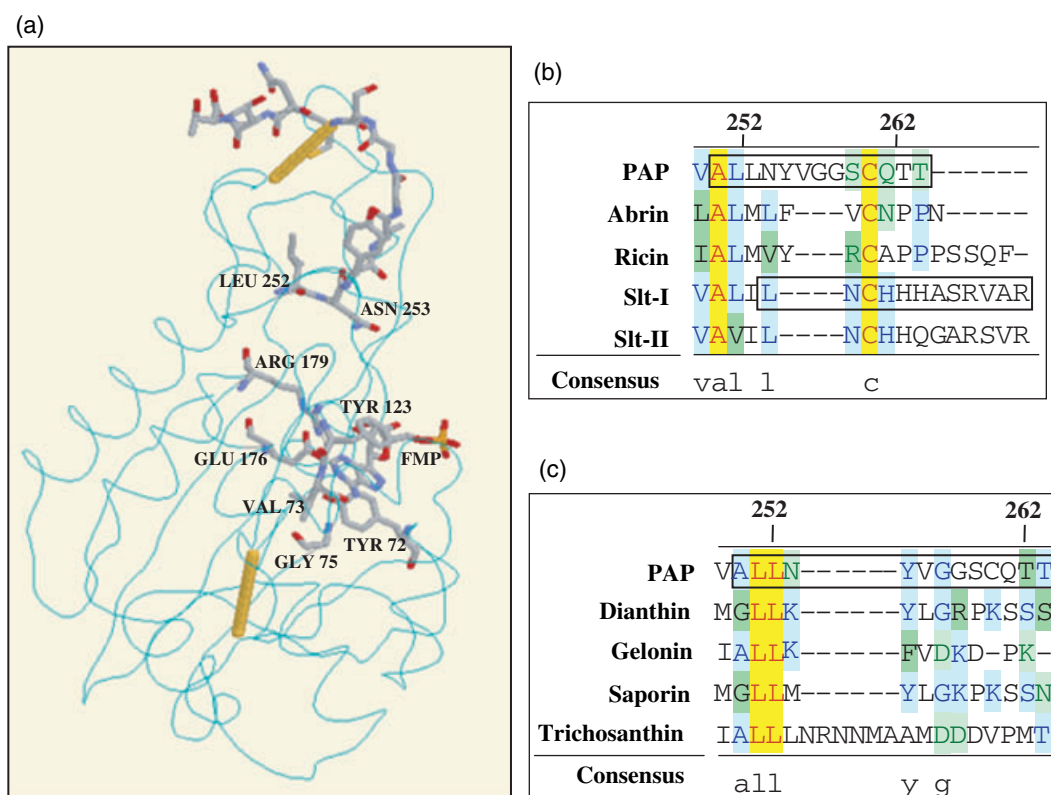


Figure 9. Comparison of the C-termini of PAP with type II and type I RIPs.

(a) The crystal structure of PAP complexed with formycin monophosphate (PDB 1PAG). The residues at the active site and the 11-amino-acid hydrophobic peptide at the C-terminus are shown.

(b) Alignment of the C-terminal sequences of mature PAP (GenBank® accession AAS48199) with the C-terminal sequences of the A chains of type II RIPs, abrin (GenBank® accession X54873), ricin (GenBank® accession AAA72820), Shiga-like toxin I (Slt-I) (GenBank® accession CAA85368) and Shiga-like toxin II (Slt-II) (GenBank® accession AAM90979). The residues critical for retrotranslocation of PAP and Shiga-like toxin I (LaPointe *et al.*, 2005) are boxed.

(c) Alignment of the C-terminal sequences of mature PAP with type I RIPs dianthin (GenBank® accession CAA41953), gelonin (GenBank® accession AAB47013), saporin (GenBank® accession CAA41948) and trichosanthin (GenBank® accession AAA34206). The residues critical for retrotranslocation of PAP are boxed.

localization of the precursor and the mature form of PAP in the ER in yeast, they do not rule out transport beyond the ER. Using a series of PAP mutants, we identified the signal critical for accumulation of PAP in the cytosol and demonstrated that distinct sequences at the C-terminus of the mature protein mediate transport, ribosome depurination and cytotoxicity. The sequence identified contains a peptide motif, which is conserved among the ribosome inactivating AB toxins and other type I RIPs, suggesting that it may act as the recognition signal for export of RIPs into the cytosol.

Experimental procedures

Constructs

Expression of PAP and PAP mutants was under the control of the *GAL1* promoter in the Yep351-based high-copy plasmid. For transformation W303 (*MATa ade2-1 trp1-1 ura3-1 leu2-3, 112 his3-11, 15 can1-100*) was grown in standard 1% yeast extract, 2% peptone and 2% dextrose (YPD) medium and transformed with Yep351 containing the mutated PAP sequences. Transformants were selected on

synthetic medium minus leucine (SD-Leu) supplemented with 2% dextrose as a carbon source.

Analysis of protein expression

Protein was extracted from frozen yeast cells harvested during the time course of induction as previously described (Hudak *et al.*, 1999). Total protein from each time point was separated on a 15% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with polyclonal anti-PAP IgG. Pokeweed antiviral protein was visualized by chemiluminescence using the Renaissance kit (PerkinElmer Life Sciences, <http://www.perkinelmer.com/>). The blots were then stripped and reprobated with monoclonal antibody against dolichol phosphate mannose synthase (Dpm1p; Invitrogen, <http://www.invitrogen.com/>) or 3-phosphoglycerate kinase (Pgk1p; Invitrogen).

Subcellular fractionation of yeast cells

Pokeweed antiviral protein-expressing yeast cells (100 ml) were grown overnight and then shifted to galactose for 4, 6, 10 or 24 h. The cells were spun down, washed once with water and lysed in an equal volume of low-salt (LS) buffer as previously described (Frey

et al., 2001). Subcellular fractionation was carried out as previously described (Parikh *et al.*, 2005).

Proteinase K protection

Microsomes were prepared and proteinase K sensitivity was assayed as previously described (Parikh *et al.*, 2005). Yeast cells (30 OD₆₀₀ units) were grown to an early log phase and were harvested by centrifugation at 1000 *g* for 5 min. The cell pellet was resuspended to 50 OD₆₀₀ ml⁻¹ in freshly made 100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl pH 9.4, 10 mM DTT for 10 min at room temperature. The cells were pelleted again and resuspended to 50 OD₆₀₀ ml⁻¹ in 1.2 M sorbitol, 50 mM potassium acetate and 20 mM TRIS-HCl pH 7.5, and 1 mg ml⁻¹ Lyticase (Sigma, <http://www.sigmaaldrich.com/>) was added. The spheroplasts were pelleted at 2000 *g* for 5 min and resuspended to 20 OD₆₀₀ ml⁻¹ in 0.1 M sorbitol, 50 mM potassium acetate, 20 mM TRIS-HCl, pH 7.5, and 1 mM phenyl-methylsulfonyl fluoride (PMSF). The spheroplasts were lysed by 15 strokes of a Dounce homogenizer. The unbroken cells were removed by centrifugation at 1000 *g* for 5 min. Microsomes were pelleted at 21 000 *g* for 10 min. The pellet was solubilized in microsome solubilization buffer (MSB; 250 mM sorbitol, 50 mM potassium acetate, 20 mM TRIS-HCl, pH 7.5). To assay for proteinase sensitivity, 20 µl of the membrane suspension was treated with or without 100 µg ml⁻¹ proteinase K (Fisher Scientific, <http://www.1.fishersci.com/index.jsp>) or 100 µg ml⁻¹ proteinase K (Fisher Scientific) and 2% TritonX-100. All treatments were performed on ice for 30 min and were stopped with 4 mM PMSF. The proteins (20 µl) were separated on a 15% SDS-polyacrylamide gel and subjected to immunoblot analysis using polyclonal anti-PAP IgG, anti-Kar2p and anti-Dpm1p.

Viability analysis

Viability was assessed by plating cells on non-inducing SD-Leu glucose plates after galactose induction in liquid media. A serial dilution of cells was plated using 20 µl of cells at 0.1 A₆₀₀ unit ml⁻¹ as the starting concentration. After incubation for 72 h at 30°C, the plates were photographed.

Ribosome depurination

Ribosomes were isolated (Tumer *et al.*, 1997) and ribosome depurination was assayed by primer extension analysis as described previously (Parikh *et al.*, 2002). The extent of ribosome depurination was determined by calculating the ratio of the extension products obtained using the depurination primer (Dep), which binds 73 nucleotides downstream of the depurination site to the control primer (25S), which binds 100 nucleotides downstream of the 5' end of the 25S rRNA.

In vivo [³⁵S] methionine incorporation

Yeast cells were grown to an A₆₀₀ of 0.6 in SD-Leu, -Met, 2% raffinose. Cells at an A₆₀₀ of 0.3 were then resuspended in 2% galactose for 4 or 10 h in order to induce either wild-type or mutant PAP expression. At time zero, [³⁵S] methionine was added to cells growing on galactose and methionine incorporation was determined as previously described (Hudak *et al.*, 2004; Parikh *et al.*, 2002).

Isolation of PAP mutants from yeast

Pokeweed antiviral protein and PAP mutants were isolated from 30 ml of yeast cells following 6 h of galactose induction. Minor modifications were done in the protocol originally described by Iizuka and Sarnow (1997). The cells were collected by centrifugation at 5000 *g* for 2 min and washed three times with cold Buffer A (30 mM HEPES, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, 8.5% w/v mannitol). For every 1 g of yeast cells, 1 ml of cold Buffer A/PMSF (0.5 mM PMSF) and 3 g of glass beads (0.5 µm) were added. The cells were broken by vortex with four 1-min bursts followed by 1 min incubation on ice. Broken cells were centrifuged at 4°C for 5 min at 22 000 *g*. The supernatants were centrifuged again at 100 000 *g* for 1 h in a Beckman tabletop ultracentrifuge to remove the ribosomes. A 2-ml Sephadex G-25 (superfine) (Amersham Biosciences, <http://www5.amershambiosciences.com>) column was packed and equilibrated with Buffer A/PMSF overnight. Yeast extract was applied to the column and the column was centrifuged at 100 *g* for 1 min. Buffer A/PMSF was added and the column was centrifuged at 100 *g* for another minute. The flow-through was collected and stored at -80°C.

Structural analysis of conserved residues

Crystal structure of PAP from the Protein Data Bank was used in conjunction with Protein Explorer v1.982, ConSurf server (Glaser *et al.*, 2003), Jellyfish and CLUSTALW (<http://www.ebi.ac.uk/clustalw>). The structure used was a PAP complex with formycin 5'-monophosphate (PDB 1PAG) (Monzinger *et al.*, 1993).

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References

- Barbieri, L., Battelli, M.G. and Stirpe, F. (1993) Ribosome-inactivating proteins from plants. *Biochim. Biophys. Acta*, **1154**, 237–282.
- Bolognesi, A. and Polito, L. (2004) Immunotoxins and other conjugates: pre-clinical studies. *Mini. Rev. Med. Chem.* **4**, 563–583.
- Bonifacino, J.S., Cosson, P., Shah, N. and Klausner, R.D. (1991) Role of potentially charged transmembrane residues in targeting proteins for retention and degradation within the endoplasmic reticulum. *EMBO J.* **10**, 2783–2793.
- Deeks, E.D., Cook, J.P., Day, P.J., Smith, D.C., Roberts, L.M. and Lord, J.M. (2002) The low lysine content of ricin A chain reduces the risk of proteolytic degradation after translocation from the endoplasmic reticulum to the cytosol. *Biochemistry*, **41**, 3405–3413.
- Di Cola, A., Frigerio, L., Lord, J.M., Ceriotti, A. and Roberts, L.M. (2001) Ricin A chain without its partner B chain is degraded after retrotranslocation from the endoplasmic reticulum to the cytosol in plant cells. *Proc. Natl Acad. Sci. USA*, **98**, 14726–14731.
- Endo, Y., Mitsui, K., Motizuki, M. and Tsurugi, K. (1987) The mechanism of action of ricin and related toxic lectins on eukaryotic

- ribosomes. The site and the characteristics of the modification in 28S ribosomal RNA caused by the toxins. *J. Biol. Chem.* **262**, 5908–5912.
- FitzGerald, D.J., Kreitman, R., Wilson, W., Squires, D. and Pastan, I. (2004) Recombinant immunotoxins for treating cancer. *Int. J. Med. Microbiol.* **293**, 577–582.
- Frankel, A.E., Kreitman, R.J. and Sausville, E.A. (2000) Targeted toxins. *Clin. Cancer Res.* **6**, 326–334.
- Fraser, M.E., Fujinaga, M., Cherney, M.M., Melton-Celsa, A.R., Twiddy, E.M., O'Brien, A.D. and James, M.N. (2004) Structure of shiga toxin type 2 (Stx2) from *Escherichia coli* O157:H7. *J. Biol. Chem.* **279**, 27511–27517.
- Frey, S., Pool, M. and Seedorf, M. (2001) Scp160p, an RNA-binding, polysome-associated protein, localizes to the endoplasmic reticulum of *Saccharomyces cerevisiae* in a microtubule-dependent manner. *J. Biol. Chem.* **276**, 15905–15912.
- Glaser, F., Pupko, T., Paz, I., Bell, R.E., Bechor-Shental, D., Martz, E. and Ben-Tal, N. (2003) ConSurf: identification of functional regions in proteins by surface-mapping of phylogenetic information. *Bioinformatics*, **19**, 163–164.
- Hartley, M.R. and Lord, J.M. (2004) Cytotoxic ribosome-inactivating lectins from plants. *Biochim. Biophys. Acta*, **1701**, 1–14.
- Hayashi, S. and Wu, H.C. (1990) Lipoproteins in bacteria. *J. Bioenerg. Biomembr.* **22**, 451–471.
- Hudak, K.A., Dinman, J.D. and Tumer, N.E. (1999) Pokeweed antiviral protein accesses ribosomes by binding to L3. *J. Biol. Chem.* **274**, 3859–3864.
- Hudak, K.A., Parikh, B.A., Di, R., Baricevic, M., Santana, M., Sesar, M. and Tumer, N.E. (2004) Generation of pokeweed antiviral protein mutations in *Saccharomyces cerevisiae*: evidence that ribosome depurination is not sufficient for cytotoxicity. *Nucleic Acids Res.* **32**, 4244–4256.
- Iizuka, N. and Sarnow, P. (1997) Translation-competent extracts from *Saccharomyces cerevisiae*: effects of L-A RNA, 5[primes] cap, and 3[primes] poly(A) tail on translational efficiency of mRNAs. *Methods*, **11**, 353–360.
- Kirst, M.E., Meyer, D.J., Gibbon, B.C., Jung, R. and Boston, R.S. (2005) Identification and characterization of endoplasmic reticulum-associated degradation proteins differentially affected by endoplasmic reticulum stress. *Plant Physiol.* **138**, 218–231.
- Knight, B. (1979) Ricin – a potent homicidal poison. *Br. Med. J.* **1**, 350–351.
- LaPointe, P., Wei, X. and Garipey, J. (2005) A role for the protease-sensitive loop region of Shiga-like toxin 1 in the retrotranslocation of its A1 domain from the endoplasmic reticulum lumen. *J. Biol. Chem.* **280**, 23310–23318.
- Lodge, J.K., Kaniewski, W.K. and Tumer, N.E. (1993) Broad-spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein. *Proc. Natl Acad. Sci. USA*, **90**, 7089–7093.
- Lord, J.M., Deeks, E., Marsden, C.J., Moore, K., Pateman, C., Smith, D.C., Spooner, R.A., Watson, P. and Roberts, L.M. (2003) Retrograde transport of toxins across the endoplasmic reticulum membrane. *Biochem. Soc. Trans.* **31**, 1260–1262.
- Martinez, I.M. and Chrispeels, M.J. (2003) Genomic analysis of the unfolded protein response in *Arabidopsis* shows its connection to important cellular processes. *Plant Cell*, **15**, 561–576.
- Monzingo, A.F., Collins, E.J., Ernst, S.R., Irvin, J.D. and Robertus, J.D. (1993) The 2.5 Å structure of pokeweed antiviral protein. *J. Mol. Biol.* **233**, 705–715.
- Muller, J., Piffanelli, P., Devoto, A., Miklis, M., Elliott, C., Ortmann, B., Schulze-Lefert, P. and Panstruga, R. (2005) Conserved ERAD-like quality control of a plant polytopic membrane protein. *Plant Cell*, **17**, 149–163.
- Nielsen, K. and Boston, R.S. (2001) Ribosome inactivating proteins: a plant perspective. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 785–816.
- Parikh, B.A. and Tumer, N.E. (2004) Antiviral activity of ribosome inactivating proteins in medicine. *Mini Rev. Med. Chem.* **4**, 523–543.
- Parikh, B.A., Coetzer, C. and Tumer, N.E. (2002) Pokeweed antiviral protein regulates the stability of its own mRNA by a mechanism that requires depurination but can be separated from depurination of the alpha-sarcin/ricin loop of rRNA. *J. Biol. Chem.* **277**, 41428–41437.
- Parikh, B.A., Baykal, U., Di, R. and Tumer, N.E. (2005) Evidence for retro-translocation of pokeweed antiviral protein from endoplasmic reticulum into cytosol and separation of its activity on ribosomes from its activity on capped RNA. *Biochemistry*, **44**, 2478–2490.
- Paton, J.C. and Paton, A.W. (1998) Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clin. Microbiol. Rev.* **11**, 450–479.
- Rajamohan, F., Pugmire, M.J., Kurinov, I.V. and Uckun, F.M. (2000) Modeling and alanine scanning mutagenesis studies of recombinant pokeweed antiviral protein. *J. Biol. Chem.* **275**, 3382–3390.
- Rapak, A., Faines, P.O. and Olsnes, S. (1997) Retrograde transport of mutant ricin to the endoplasmic reticulum with subsequent translocation to cytosol. *Proc. Natl Acad. Sci. USA*, **94**, 3783–3788.
- Ready, M.P., Brown, D.T. and Robertus, J.D. (1986) Extracellular localization of pokeweed antiviral protein. *Proc. Natl Acad. Sci. USA*, **83**, 5053–5056.
- Richardson, P.T., Westby, M., Roberts, L.M., Gould, J.H., Colman, A. and Lord, J.M. (1989) Recombinant proricin binds galactose but does not depurinate 28S ribosomal RNA. *FEBS Lett.* **255**, 15–20.
- Sandvig, K. and van Deurs, B. (2005) Delivery into cells: lessons learned from plant and bacterial toxins. *Gene Ther.* **12**, 865–872.
- Sandvig, K., Garred, O., Prydz, K., Kozlov, J.V., Hansen, S.H. and van Deurs, B. (1992) Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature*, **358**, 510–512.
- Simpson, J.C., Lord, J.M. and Roberts, L.M. (1995) Point mutations in the hydrophobic C-terminal region of ricin A chain indicate that Pro250 plays a key role in membrane translocation. *Eur. J. Biochem.* **232**, 458–463.
- Simpson, J.C., Roberts, L.M., Romisch, K., Davey, J., Wolf, D.H. and Lord, J.M. (1999) Ricin A chain utilizes the endoplasmic reticulum-associated protein degradation pathway to enter the cytosol of yeast. *FEBS Lett.* **459**, 80–84.
- Sixma, T.K., Kalk, K.H., van Zanten, B.A., Dauter, Z., Kingma, J., Witholt, B. and Hol, W.G. (1993) Refined structure of *Escherichia coli* heat-labile enterotoxin, a close relative of cholera toxin. *J. Mol. Biol.* **230**, 890–918.
- Stirpe, F. (2004) Ribosome-inactivating proteins. *Toxicon*, **44**, 371–383.
- Suhan, M.L. and Hovde, C.J. (1998) Disruption of an internal membrane-spanning region in Shiga toxin 1 reduces cytotoxicity. *Infect Immun.* **66**, 5252–5259.
- Tumer, N.E., Hwang, D.J. and Bonness, M. (1997) C-terminal deletion mutant of pokeweed antiviral protein inhibits viral infection but does not depurinate host ribosomes. *Proc. Natl Acad. Sci. USA*, **94**, 3866–3871.
- Wang, P. and Tumer, N.E. (2000) Virus resistance mediated by ribosome inactivating proteins. *Adv. Virus Res.* **55**, 325–355.